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Ethanol and protein production from minimally processed biomass of a genetically-modified cyanobacterium over-accumulating sucrose

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ABSTRACT

One of the main bottlenecks of a microalgal or cyanobacterial biomass biorefinery is the separation of different useful fractions using simple, low energy-consuming, cost-effective, and scalable separation processes. Although the carbohydrates-rich biomass of these microorganisms presents clear advantages over conventional terrestrial crops as feedstocks for ethanol, it still requires acid and/or enzymatic hydrolysis for efficient fermentation. Here, we show the genetic modification of carbohydrates partitioning in a filamentous cyanobacterium towards the accumulation of sucrose up to 10% (w/w) as a readily fermentable feedstock. We optimized two methods for the preparation of concentrated sucrose syrups, which were efficiently converted into ethanol by yeasts, without the need of additional pretreatments. Biomass drying and milling, followed by aqueous extraction of sugars and proteins, and the recovery of proteins by short pulses of heat, kept the value of sugars as a feedstock for ethanol and protein for feed supplements within a cost-effective biomass biorefinery.

1. Introduction

Most techno-economic analysis tends to indicate that costs to produce only biofuels from microalgal or cyanobacterial biomass remain too high for profitable commercialization. Similar analyses also suggest that co-production of biofuels and animal feed, or other higher-value co-products within biomass biorefineries, would largely improve cost-effectiveness (Laurens et al., 2017).

In addition to aspects directly related to the cost of producing biomass (Luan and Lu, 2018), the main bottleneck for a biorefinery approach is the separation of different useful fractions using simple, low energy-consuming, cost-effective, and scalable separation processes (Chew et al., 2017; Luan and Lu, 2018).

Microalgal and/or cyanobacterial carbohydrates present considerable advantages over plant-based feedstocks for the production of ethanol (Sanz Smachetti et al., 2018). The lack of lignin and its simpler structure tend to make the pretreatment and saccharification of microalgae or cyanobacterial polysaccharides less energy-intensive and reagent-demanding than current plant-based feedstocks (Sanz Smachetti et al., 2018). It has been shown that microalgal and cyanobacterial carbohydrate–rich biomass can be efficiently hydrolyzed into monosaccharides by chemical (acid or alkaline)/physicochemical or enzymatic hydrolysis (Hernández et al., 2015). While acid hydrolysis, mostly using diluted sulfuric acid, requires high temperatures and produces large volume of contaminating residues, enzymatic hydrolysis can be completed under milder temperatures, although it still requires some physical or chemical pretreatments, and it is considerably more expensive (Hernández et al., 2015). Nevertheless, both strategies lead to biomass saccharification and conversion into ethanol by microbial fermentation with an efficiency very close to the upper theoretical value, as recently compiled by Sanchez Rizza and co-workers (Sanchez Rizza et al., 2017; Sanz Smachetti et al., 2018).

Both microalgae and cyanobacteria can also accumulate soluble sugars, mainly in response to salt, osmotic, desiccation, cold, or heat stress (Kolman et al., 2015; Salerno et al., 2004). In cyanobacteria, the predominant soluble carbohydrates that accumulate upon salt stress are the disaccharides sucrose and trehalose, glucosylglycerol and glucosylglycerate, and sucroglucans (Kolman et al., 2015; Pontis et al., 2007). Sucrose is the most generalized one, and in addition to its function as a compatible solute during environmental stress, it also appears to display other functions in diazotrophic growth and/or in cell signaling pathways in some strains (Cumino et al., 2007; Curatti et al., 2002; Desplats et al., 2005; Kolman et al., 2015). Sucrose metabolism in cyanobacteria is similar to that of microalgae and plants: it is synthesized by the sequential action of sucrose-phosphate synthase (SPS, U/ADP-glucose: D-fructose-6-phosphate 2-α-D-glucosyltransferase, EC 2.4.1.14) and sucrose-phosphate phosphatase (SPP, sucrose-6F-phosphate-phosphohydrolase, EC 3.1.3.24); while its catabolism is initiated

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by the activity of three different enzymes: (*i*) sucrose synthase (SuS, A/UDP-glucose: D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13); (*ii*) alkaline/neutral invertase (A/N-Inv, an α-glycosidase, EC 3.2.1.261); and (*iii*) amylosucrase (AMS, EC 2.4.1.4) (Kolman et al., 2015; Salerno and Curatti, 2003).

Soluble carbohydrates such as glucose or sucrose are very attractive carbon sources because they are readily up-taken by yeasts and most microorganism of industrial importance to produce ethanol and other fermentation products without the need of any of the pretreatments already described (Marques et al., 2016; Verstrepen et al., 2004). Thus, during the last years, much attention has been devoted to the genetic modification of unicellular cyanobacteria to increase biosynthesis and release of glucose or sucrose into the medium as an alternative to plant crop-based carbohydrates as fermentation feedstocks (Du et al., 2013; Duan et al., 2016; Ducat et al., 2012; Hays et al., 2017; Kirsch et al., 2018; Löwe et al., 2017; Smith and Francis, 2017; Song et al., 2016; Weiss et al., 2017). Some estimations suggested that sucrose productivity of genetically modified *Synechococcus elongatus* could be several-fold higher than that of sugarcane, assuming the scalability of laboratory results on either a volumetric or areal basis (Ducat et al., 2012). These sugar-exporting strains cross-fed sugars to heterotrophic microorganisms such as the yeast *Saccharomyces cerevisiae* or bacteria when co-cultivated (Du et al., 2013; Duan et al., 2016; Ducat et al., 2012; Hays et al., 2017; Löwe et al., 2017; Smith and Francis, 2017; Weiss et al., 2017). In some cases, co-cultures allowed production of target compounds, such as polyhydroxybutyrate, from atmospheric CO_2 (Löwe et al., 2017; Smith and Francis, 2017).

In this report we show that a filamentous cyanobacterium overexpressing a sucrose-phosphate synthase encoding-gene over-accumulates sucrose intracellularly up to 10% (w/w) of its dry weight after induction by a NaCl stress. We show optimization of two low energy- and reagents-demanding methods for cell collection and preparation of sugar-enriched fractions as suitable feedstocks for ethanol fermentation. Additionally, a large fraction of protein could be recovered as a potential animal feed supplement.

2. Materials and methods

2.1. Plasmid construction and strain transformation

A mutant strain of *Anabaena* sp. PCC 7120 was obtained by cloning the native *spsB* gene (Cumino et al., 2002) in the plasmid pRL1404 in front of its own promoter. The resulting construct was transferred into the cyanobacterium by conjugation. Methods for DNA manipulation and transgenic strains isolation were described before (Curatti et al., 2002).

2.2. Strains' culture conditions

Anabaena sp. strain PCC 7120 and the derivative transgenic strain were routinely cultivated in BG11 medium ($0.04\ g{\cdot}L^{-1}$ K_2HPO_4; $0.075\ g{\cdot}L^{-1}$ $MgSO_4{\cdot}7H_2O$; $0.036\ g{\cdot}L^{-1}$ $CaCl_2{\cdot}2H_2O$; $0.006\ g{\cdot}L^{-1}$ citric acid; $0.006\ g{\cdot}L^{-1}$ ferric ammonium citrate; $0.001\ g{\cdot}L^{-1}$ EDTA (disodium salt); $0.02\ g{\cdot}L^{-1}$ Na_2CO_3, and trace metal mix A5 ($2.86\ mg{\cdot}L^{-1}$ H_3BO_3; $1.81\ mg{\cdot}L^{-1}$ $MnCl_2{\cdot}4H_2O$; $0.222\ mg{\cdot}L^{-1}$ $ZnSO_4{\cdot}7H_2O$; $0.39\ mg{\cdot}L^{-1}$ $NaMoO_4{\cdot}2H_2O$; $0.079\ mg{\cdot}L^{-1}$ $CuSO_4{\cdot}5H_2O$ and $0.049\ mg{\cdot}L^{-1}$ $Co(NO_3)_2{\cdot}6H_2O$)), containing $1.5\ g{\cdot}L^{-1}$ $NaNO_3$ as a nitrogen source. For isolation and maintenance of the transgenic strain, streptomycin and neomycin were used at $5\ mg{\cdot}L^{-1}$ and $150\ mg{\cdot}L^{-1}$, respectively.

Strains were maintained in BG11-agar Petri dishes illuminated with constant white light at 6 μmol photons $m^{-2}{\cdot}s^{-1}$. For inoculum preparation, strains were first cultivated in 100 mL Erlenmeyer flasks containing 20 mL of BG11 medium supplemented with $2.4\ g{\cdot}L^{-1}$ Hepes-NaOH, pH 7.5; with constant agitation at 120 rpm and illuminated with constant white light at 30 μmol photons $m^{-2}{\cdot}s^{-1}$. Then, cultures were inoculated into 500 mL bottles containing 300 mL of BG11 medium supplemented with $8.4\ g{\cdot}L^{-1}$ $NaHCO_3$ and sparged from the bottom with filtered CO_2-enriched air (at about 5000 ppm) at 0.3–0.5 $L{\cdot}min^{-1}$ and illuminated with constant white light at 75 μmol photons $m^{-2}{\cdot}s^{-1}$. Sucrose accumulation experiments were carried out in 5 L air-lift column photobioreactors (PBRs) containing 4.5 L of BG11 supplemented with $8.4\ g{\cdot}L^{-1}$ $NaHCO_3$, sparged with air from the center of the riser tube at $6\ L{\cdot}min^{-1}$ (up flow circulation) and with pure CO_2 from the bottom of the down flow circulation at $0.2\ L{\cdot}min^{-1}$, and illuminated with constant white light at 213.8 μmol photons $m^{-2}{\cdot}s^{-1}$. Temperature was maintained constant at 28 ± 1 °C in all cases. As indicated in place, cultures were supplemented with 80 mM or 120 mM NaCl.

Cells used to determine SPS activity were cultivated in 250 mL Erlenmeyer flasks containing 50 mL of BG11 medium with constant agitation at 120 rpm and illuminated with constant white light at 30 μmol photons $m^{-2}{\cdot}s^{-1}$.

For fermentation analysis, the yeast *Saccharomyces cerevisiae* (Levex®, Argentina) was maintained in YPD-agar containing 1% yeast extract, 2.5% peptone, 2% dextrose and 1% agar-agar, at 28 °C in the dark.

2.3. Biomass processing and sugars extraction

To obtain cyanobacterial sugar-rich extracts, 4.5 L of *Anabaena* sp. PCC 7120 *wt* or *spsB*$^+$ cultures were promptly cooled down by adding crashed ice and supplemented with $240\ mg{\cdot}L^{-1}$ $FeCl_3$ to promote flocculation. The decanted-cells slurry was further dewatered by centrifugation at 6000 ×*g*. For the sugars extraction-method based on microwaves (MW), the cell paste was subjected to extraction by microwaves at 200 W of power, for 4 cycles of 2 min each, in a microwave oven (BGH Quick Chef® 15140, Argentina) and the soluble fraction was separated by centrifugation at 17,211 ×*g* for 15 min. The removed volume was replaced with distilled water and centrifuged once again. Both soluble fractions were combined.

For the sugars extraction-method based on dry milling of the biomass (D&M), the dewatered biomass was air-dried, milled with 15% sand (w/w) and rehydrated with water at a 1:3.5 ratio (w/v). Glass beads were added and then, vigorously vortexed. After centrifugation at 11,952 ×*g* for 15 min, the soluble fraction was separated, and the removed volume was replaced with distilled water. This step was repeated 3 times. All soluble fractions were combined and incubated at 100 °C for 5 min to partially remove the soluble proteins. Incubation conditions that ensure maximum protein precipitation and minimal sucrose lost were chosen by incubating the extracts at 60 or 100 °C for 5, 10, 20 and 40 min, and protein and sucrose content in the extract was determined (Supplementary Materials). Regardless the method of extraction, soluble fractions were stored at −20 °C until further analysis.

2.4. Fermentation

Sucrose-rich extracts from *Anabaena* sp. PCC 7120 *wt* or *spsB*$^+$ biomass were taken to pH 6.5 with H_2SO_4 and supplemented with $19\ g{\cdot}L^{-1}$ $MgSO_4$. Micro-fermentations (1 mL) were conducted as previously described (Sanchez Rizza et al., 2017) by inoculating the sucrose-rich preparation with *S. cerevisiae* cells at an initial OD_{600} of 0.25 for 24 or 48 h at 28 °C and agitation at 120 rpm. Samples were taken periodically, centrifuged at 16,300 ×*g* for 5 min, and supernatants were stored at −20 °C until further analysis. As a positive control, fermentation of YPD medium, at a dextrose concentration in the range of sucrose content in the samples, was routinely conducted.

2.5. Analytical methods

For dry weight determinations, 50 mL of resuspended cells in culture medium were first centrifuged at 3900 ×*g* for 10 min, transferred into a 1.5 mL centrifuge tube and then, centrifuged at 16,300 ×*g* for

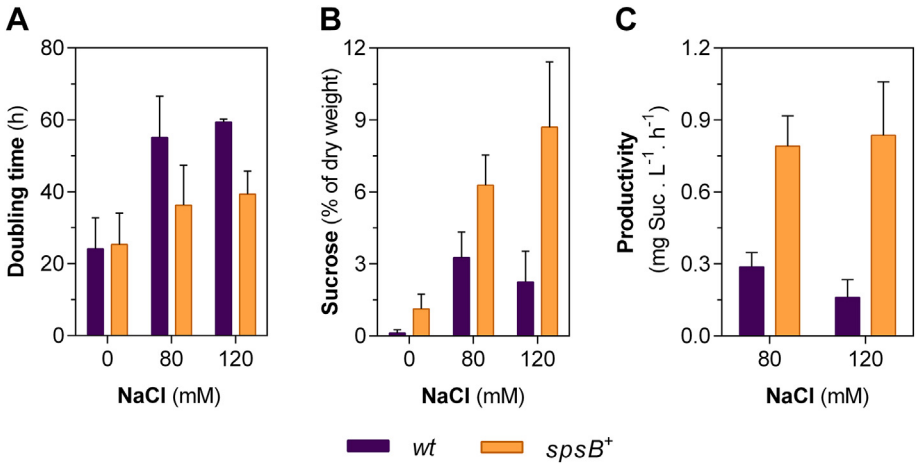

Fig. 1. Effect of *spsB* gene overexpression on growth and sucrose accumulation. A) Cyanobacterial strains doubling time in the presence of different concentrations of NaCl. Data at 0 and 80 mM NaCl represent the mean and SD of five to eight independent experiments; and at 120 mM, the mean and SD of two independent experiments. B) Sucrose content, as percentage of dry biomass, of cells cultured in the presence of different concentrations of NaCl. Data at 0 and 80 mM NaCl represent the mean and SD of four to ten independent experiments; and at 120 mM, the mean and SD of two to four independent experiments. C) Sucrose productivity of *Anabaena* sp. strains cultured at different concentrations of NaCl. Data at 80 mM NaCl represent the mean and SD of five or six independent experiments; and at 120 mM, the mean and SD of two to four independent experiments.

5 min, at 4 °C. Pellets were dried out in an oven at 90 °C until constant weight (2–3 days).

For growth curves analysis, cell density was estimated by recording periodically OD at 750 nm in a UV-1800 spectrophotometer (Shimadzu, Japan). Data were plotted using the GraphPad PRISM software (Intuitive Software for Science, US) and doubling times were obtained by fitting the experimental data to theoretical curves of exponential growth with R^2 above 0.96 (Supplementary Materials). Doubling time was also calculated from dry weight data for an observed correspondence below 10% difference between both methods.

For protein determination, samples of the sucrose-rich extracts were subjected to the Lowry's method (Lowry et al., 1951), using bovine serum albumin as a standard. Alternatively, crude protein was calculated after the combustion of the samples in an atmosphere of ultrapure O_2 and helium at 850 °C, total N was determined in a LECO FP 528 system, using EDTA as a calibration standard, and the standard N-to-protein conversion factor 4.44, characteristic of cyanobacterial protein, was applied (González López et al., 2010).

Total lipids were determined gravimetrically after lipids extraction, basically according to Bligh and Dyer (1959) with modifications (Do Nascimento et al., 2012).

For total carbohydrates determination, samples were reacted with the anthrone reagent (Dreywood, 1946). Standard sucrose extraction and determination were conducted essentially as reported (Pontis, 2017). Briefly, 50 mL of *Anabaena* sp. PCC 7120 *wt* or *spsB*$^+$ culture were centrifuged at 3600 ×*g* for 15 min, at 4 °C. Cells were resuspended in 2 volumes of boiling alkaline water (pH 8), incubated at 100 °C for 5 min and then, centrifuged at 9600 ×*g* for 5 min, at 4 °C. Extraction was repeated two more times and the fractions were combined and stored at −20 °C until further analysis. For sucrose determination, samples in 100 mM NaOAc, pH 4.5 buffer were incubated at 55 °C in the presence of 80 μg·mL^{-1} acid invertase (Sigma-Aldridge) for 30 min for its conversion into glucose and fructose, which were later determined by the Somogyi-Nelson's method with a standard curve using sucrose (Pontis, 2017).

For ethanol determination, yeast spent medium after fermentation was subjected to a method previously reported (Sanchez Rizza et al., 2017). Briefly, the standard ethanol assays contained 50 mM Tris-HCl, pH 8.4; 2.5 mM NAD^+ and 3 μg protein preparations containing alcohol dehydrogenase activity and the samples in a total volume of 100 μL and were incubated at 30 °C for 25 min. Ethanol was determined as the ethanol-dependent reduction of NAD^+ at 340 nm and compared with a standard curve made with 99% (v/v) analytical grade ethanol.

SPS activity was assayed essentially as reported before (Porchia and Salerno, 1996). Protein extracts were prepared from cells collected from 50 mL of culture. Cells were resuspended in 3 volumes (fw/v) of a buffer containing 100 mM Hepes-NaOH, pH 7.5; 50 μM PMSF; 2 mM EDTA; 20 mM $MgCl_2$; 2% (v/v) ethylene glycol; 20 mM 2-mercaptoethanol; and 20% glycerol, frozen under liquid N_2 and milled in a drill in the presence of acid-washed glass dust. After clarification by centrifugation at 9600 ×*g* for 20 min at 4 °C, protein extracts were desalted through BioGel P10 resin, previously equilibrated in the same buffer. SPS activity assays, containing 100 mM Hepes-NaOH, pH 7.5; 10 mM Fru-6-P; 10 mM UDP-Glc; 20 mM $MgCl_2$; 50 mM NaF; and 5 mM arbutin, were incubated at 30 °C. The production of sucrose-6-P was determined by the thiobarbituric acid method, using fructose as a standard (Pontis, 2017).

3. Results and discussion

3.1. Genetic modification of sucrose accumulation in Anabaena sp. PCC 7120

We constructed an *Anabaena* strain over-expressing the *spsB* gene as an alternative platform for producing readily fermentable sugars. Thus, we placed the native *Anabaena* sp. PCC 7120 *spsB* gene, encoding a SPS enzyme (Cumino et al., 2002) and its putative promoter, downstream of the constitutive promoter of pDU1 of the shuttle vector pRL1404.

The transgenic strain showed an average increase of SPS activity of 2-fold, from 1 nmol Fru. mg prot.$^{-1}$·min^{-1} to 2 nmol Fru. mg prot.$^{-1}$·min^{-1}, when cells were cultivated under standard growth conditions. Under this culture condition, the transgenic strain showed no growth difference when compared to the parental strain, with doubling times of 25.3 ± 8.7 h^{-1} and 24.1 ± 8.6 h^{-1}, respectively. However, the transgenic line was more tolerant to NaCl loading in the culture medium, especially at 80 mM NaCl, displaying a doubling time of 36.2 ± 11.1 h^{-1}, in comparison to 55.1 ± 11.4 h^{-1} of the wild type strain (Fig. 1A and Supplementary Materials).

As expected, under standard growth conditions, the mutant strain accumulated 10-fold more sucrose in its biomass than the parental strain. Under saline stress at 80 mM or 120 mM NaCl for 48 h, sucrose content of the transgenic line was 2- or 4-fold higher than the wild type, reaching values of 6.3 ± 1.3 (w/w) or 8.7 ± 2.7% (w/w) of their dry biomass, respectively (Fig. 1B). However, the overall sucrose productivity was offset by slower growth under more stringent stressing conditions for a similar productivity at around 0.8 mg Suc. L^{-1}·h^{-1} (Fig. 1C).

Fig. 2 shows that biomass of both *Anabaena* strains presented similar levels of total carbohydrates at about 27% (w/w) (Fig. 2A). However, while sucrose represented a minor fraction of total carbohydrates of non-stressed cells of both strains, its level increased up to 43.6 ± 9.7% (w/w) of total biomass carbohydrates in the transgenic strain (Fig. 2D).

Thus, NaCl stress appears to exert a major shift in carbohydrates partitioning, which can be exacerbated by over-expression of a SPS

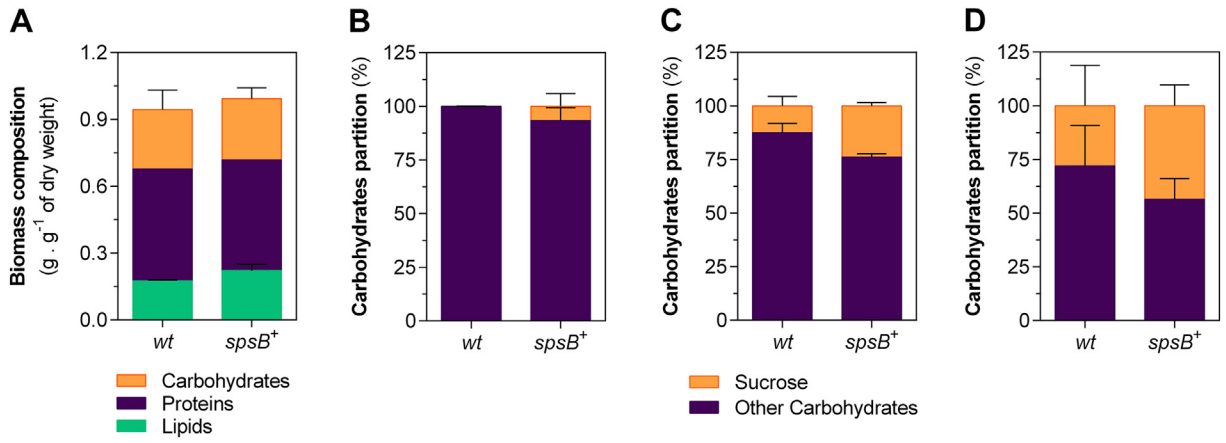

Fig. 2. Effect of *spsB* gene overexpression on biomass composition and carbohydrates partitioning. A) Total carbohydrates, total proteins and total lipids content after 48 h of induction with 80 mM NaCl. For carbohydrates, proteins and lipids, data represent the mean and SD of four, independent experiments; a single determination obtained from the pool of four independently obtained samples; and two independent experiments, respectively. B–D) Soluble carbohydrates partitioning between sucrose and other carbohydrates at 0 (B), 24 (C) and 48 (D) h of induction with 120 mM NaCl. Data represent the mean and standard deviation of two independent experiments (B and C) or four independent experiments (D).

encoding-gene. Interconnection between the glycogen and sucrose pools has been demonstrated in some unicellular cyanobacteria, (Ducat et al., 2012; Miao et al., 2003; Qiao et al., 2018; Suzuki et al., 2010; Xu et al., 2013) and also in *Anabaena* spp. (Curatti et al., 2008). Although it has been generally interpreted that sucrose and glycogen synthesis were competing pathways at the levels of the common substrate glucose-1-phosphate, a recent study in *S. elongatus* PCC 7942 showed that glycogen synthesis was required for NaCl induction of sucrose accumulation (Qiao et al., 2018). This result suggested that, in addition to mutation of genes for sucrose breakdown (Ducat et al., 2012; Kirsch et al., 2018), metabolic engineering of glycogen synthesis and mobilization could serve as an additional source of glucose-1-phosphate to further boost sucrose accumulation in *sps* genes over-expressing strains.

It has been pointed out that in order for cyanobacteria to be utilized successfully as biofactories, especially for commodities, tolerance to environmental stress must be increased (Kitchener and Grunden, 2018). Moreover, identifying or creating hyper-tolerant strains either to natural (or even artificially enhanced) stressing conditions, might represent a crop protection strategy by discouraging competitors and/or predators. Sucrose accumulation upon salt stress has long been demonstrated in *Anabaena* spp. as well as in other cyanobacteria. This has led to the general view that it serves the function of an osmolite enabling cells to stand salt stressing conditions (Kolman et al., 2015; Kirsch et al., 2018). Both sucrose accumulation under osmotic stress and/or high temperatures have led to identical interpretations (Warr et al., 1985). Kitchener and Grunden (2018), discussed the challenge of diverting cellular resources towards an efficient stress tolerance response alongside maximizing accumulation of the target product. The coincidental nature of sucrose as a key player of the stress tolerance response and the target product would represent a very interesting advantage of this platform.

Until this study and regardless of the vast cumulative evidence concerning sucrose accumulation upon salt stress, it has been difficult to accurately estimate up to what extent sucrose accumulation alone could promote salt stress tolerance. Salt stress tolerance is a complex process that, in addition to accumulation of osmolites, also involves active efflux of ions and change in expression of many genes (Hagemann, 2016). Our results suggest the feasibility of increasing tolerance to stress by the same kind of mutations that promote the accumulation of the target product.

3.2. Sucrose extraction from Anabaena biomass

Cyanobacterial strains were cultivated in 5 L photobioreactors and cells were efficiently collected by flocculation in the presence of $FeCl_3$ and further dewatered by mild centrifugation. Then, we optimized two methods for sugar extraction: one consisted in drying and milling (D&M) the biomass, followed by an aqueous extraction at room temperature (about 22 °C) and the other one was based on microwaves (MW) treatment of the wet biomass. Both procedures required further clarification by centrifugation. The D&M or MW methods allowed recoveries of 54.5 ± 11.3% or 84.3 ± 13.7% of the sucrose content of the transgenic strain's biomass, respectively. However, the opposite trend was consistently observed for biomass of the *wt* strain (Fig. 3). Although the reason for this difference is currently not understood, we speculate it can be related to some other pleiotropic effect of the mutation on the structure and/or biochemical composition of the biomass.

The maximum soluble-carbohydrates and sucrose content of the transgenic strain biomass, induced at 120 mM NaCl and extracted by D&M method was 21.5 ± 0.3 $g{\cdot}L^{-1}$ and 12.9 ± 1.1 $g{\cdot}L^{-1}$, respectively (Fig. 4).

As a platform for producing fermentable sugars, this alternative strategy brings some advantages over unicellular cyanobacteria overproducing and excreting sucrose. First, larger and filamentous strains, aided by flocculation, are easier to separate from the culture medium. Additionally, sugar retention in the biomass and simplified biomass

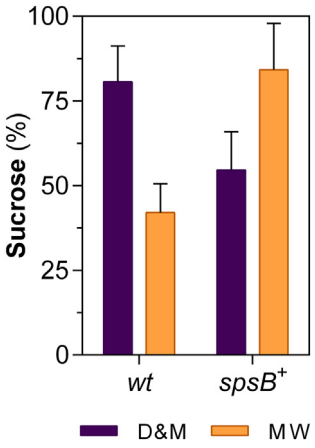

Fig. 3. Biomass sucrose extraction by two alternative methods. D&M and MW methods. Sucrose extracted by the different methods was expressed as a percentage of total biomass sucrose. Data represent the mean and range of two independent experiments.

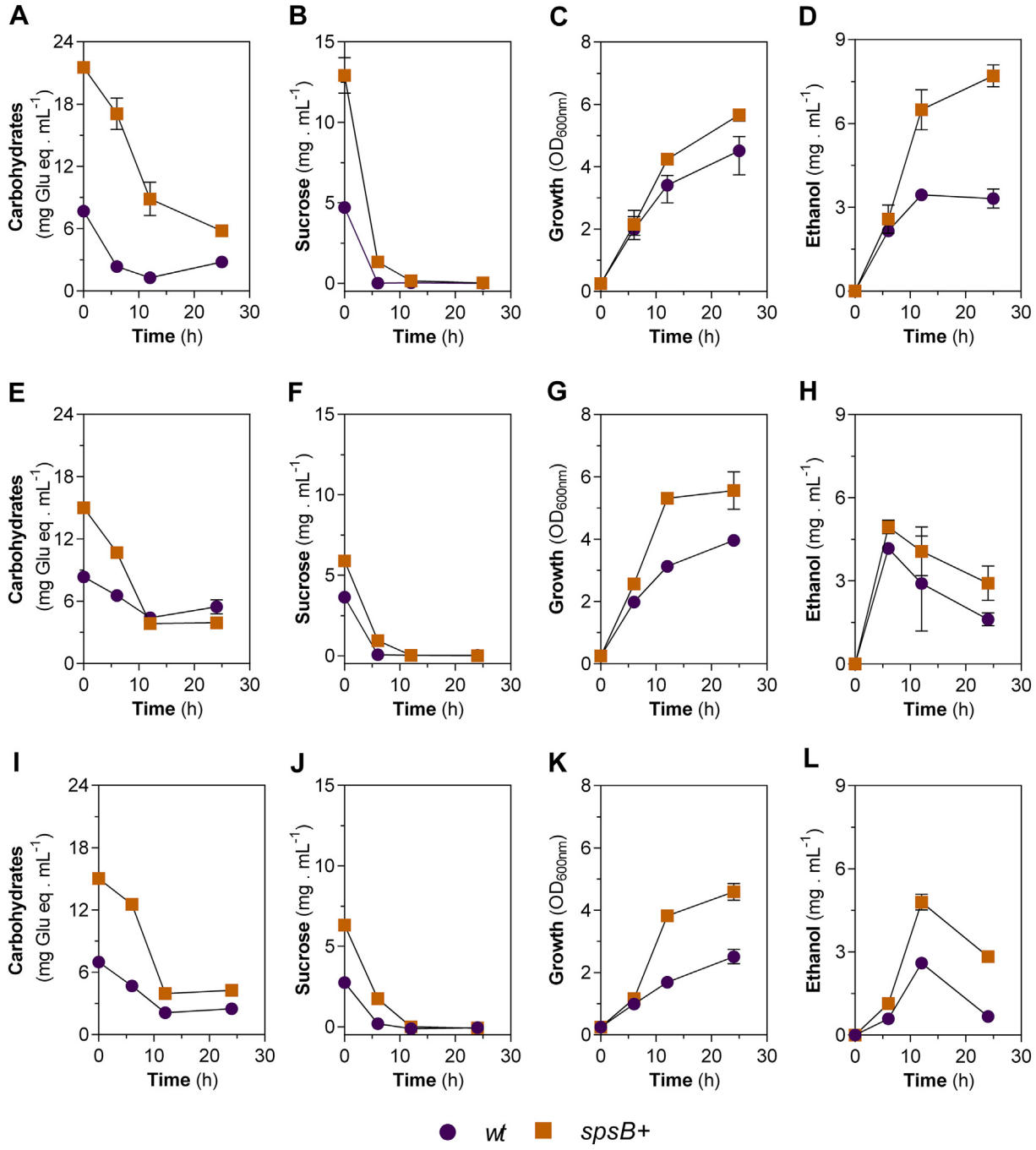

Fig. 4. Time course of the fermentation of the sucrose-rich syrups. Sucrose accumulation was induced at 120 mM (A–D) or 80 mM NaCl (E–L). Sucrose was extracted from the biomass using the D&M (A–H) or the MW (I–L) procedures. A, E and I) Depletion of total soluble carbohydrates. B, F and J) Sucrose consumption. C, G and K) Growth curve of *S. cerevisiae* (OD_{600}). D, H and L) Ethanol production. The extract obtained via the MW method was supplemented with yeast extract and peptone. The data represent the mean and range of two independent experiments.

recovery allowed us to produce more concentrated preparations of up to 22 g·L^{-1} of sugars and up to 13 g sucrose·L^{-1}. This result improves most frequent concentrations around 1.5 g sucrose·L^{-1} from sucrose exporting *Synecchococcus* sp. PCC 7942 strains (Du et al., 2013; Duan et al., 2016; Ducat et al., 2012; Hays et al., 2017; Kirsch et al., 2018; Löwe et al., 2017; Niederholtmeyer et al., 2010; Smith and Francis, 2017; Song et al., 2016; Weiss et al., 2017) and also the most recent improvement after genetic manipulation of sucrose synthesis and export, and cell proliferation at 6 g sucrose·L^{-1} (Abramson et al., 2018). This aspect may have technological implications if this kind of cyanobacteria-based platforms are to be envisioned as a source of sugars for the production of ethanol, since a minimum of 40 g ethanol·L^{-1} would be needed to reduce distillation costs (Möllers et al., 2014). In such a case, and according to the stoichiometry of ethanol fermentation, and assuming a 100% conversion, this would demand a fermentation broth containing at least 80 g sugar·L^{-1}. A recent study showed the production of up to 72.9 g sugar·L^{-1} after saccharification of a microalgal biomass with H_2SO_4 at high temperature and quantitative conversion into ethanol by *S. cerevisiae* (Sanchez Rizza et al., 2017).

The procedures optimized in this study largely improved the state-of-the-art of the production of concentrated preparations of sugars as fermentation feedstocks without the need of biomass hydrolysis with acids and/or enzymes. However, considerable improvements are still required to bust sugars concentration. These improvements may result

from a combination of additional genetic modifications and/or host strain selection, and further optimization of biomass processing.

3.3. Ethanol production from Anabaena sugar preparations

Ethanol production represents one key example of several alternative fermentation end-products using sugars syrups from cyanobacterial or microalgae. Preliminary attempts of fermenting sugar preparations obtained by the D&M procedure with *S. cerevisiae* produced low levels of ethanol (data not shown). We observed that this extraction procedure also recovered a large proportion of soluble protein at 23–29 $g{\cdot}L^{-1}$, which increased the viscosity of the extracts (not shown). Thus, we analyzed the effect of temperature on sugar and protein separation and the stability of sucrose. We observed that incubations as short as 5 min at 100 °C were enough to recover about 90% of the proteins as insoluble material while sucrose content remained unchanged (Supplementary Materials).

Deproteinized sugar preparations were quantitatively converted into ethanol by fermentation with *S. cerevisiae*. Fig. 4 shows the time course of total sugars (Fig. 4A, E and I) or sucrose (Fig. 4B, F and J) depletion, the concomitant growth of the yeast (Fig. 4C, G and K), and increase in ethanol production (Fig. 4D, H and L). Sucrose-rich extracts from biomass induced at 120 mM NaCl and processed according to D&M method (Fig. 4A–D) contained total soluble carbohydrates at 7.7 ± 0.8 $g{\cdot}L^{-1}$ or 21.6 ± 0.5 $g{\cdot}L^{-1}$ for the *wt* type or transgenic biomass, respectively. These preparations also contained sucrose at 4.7 ± 0.4 $g{\cdot}L^{-1}$ or 12.9 ± 0.5 $g{\cdot}L^{-1}$ for the *wt* type or transgenic biomass, respectively. Fermentation of these preparations produced ethanol up to 3.4 ± 0.2 $g{\cdot}L^{-1}$ or 7.7 ± 0.6 $g{\cdot}L^{-1}$ for the *wt* type or transgenic biomass, respectively (Fig. 4D). Consumed carbohydrates were converted into ethanol at least up to 89.4% or 70.2% of the maximum theoretical value of 0.51 g ethanol·g $glucose^{-1}$, from the biomass of the *wt* or the transgenic strain, respectively.

While sucrose was completely consumed by the yeast (Fig. 4B, F and J), a soluble carbohydrates fraction remained non-metabolized by *S. cerevisiae* under the used fermentation conditions (Fig. 4A, E and I). Although not experimentally demonstrated in this work, it is presumed that the carbohydrates fraction that remained non-metabolized by *S. cerevisiae* could correspond to sucroglucans. These oligosaccharides represent, after sucrose, the second most abundant fraction of the soluble carbohydrates that accumulate in *Anabaena* and *Nostoc* strains exposed to salt stress (Salerno et al., 2004). Sucroglucans share the general structure [α-D-Glc*p*-(1 → 2)]$_n$-α-D-Glc*p*-(1 → 2)-β-D-Fru*f*, and are characterized by a [α-D-Glc*p*-(1 → 2)] linkage that is quite non frequent in nature (Pontis et al., 2007). Although accumulation in cyanobacteria can be reverted upon reversal of the salt stress (Salerno et al., 2004), the catabolism of sucroglucans is largely unknown, and appeared not to involve [α-D-(1 → 4)] glucosidase, invertase or sucrase activities (Pontis et al., 2007). Thus, to further improve this platform for the production of fermentable sugars, the nature of these carbohydrates must be confirmed, and its metabolic pathways elucidated in order to design engineering strategies to further modify the partitioning of carbon in the cyanobacterium and/or to transfer appropriate genes to microorganisms to allow fermentation into products.

Similar results were obtained from cyanobacteria induced with 80 mM NaCl using the D&M (Fig. 4E–H) or the MW (Fig. 4I–L) methods, respectively. However, unlike with the D&M method, sugars prepared by the MW method, required supplementation of an additional source of nutrients such as yeast extract, for a more efficient conversion into ethanol (Figs. 4 and 5, and Supplementary Materials).

When integrating results from sucrose productivity by the cyanobacteria, efficiency of sugars extraction, and conversion into ethanol, it was observed that the transgenic strain produced about twice the ethanol per unit of biomass culture volume, regardless of the biomass processing method (Fig. 5).

Recently, an elegant study showed cell type-specific metabolic engineering of *Anabaena* sp. PCC 7120 to express *Zymomonas mobilis* pyruvate decarboxylase and *Synechocystis* sp. PCC 6803 alcohol dehydrogenase genes exclusively in heterocysts. In contrast to the O_2-evolving photosynthetic vegetative cells, heterocysts differentiate from vegetative cells upon combined N shortage and execute a complex shift in genes expression which down-regulates O_2-evolving photosynthesis and induces N_2-fixation, which is a strict anaerobic pathway. A mutant strain additionally overexpressing an invertase gene in heterocysts, and assisted by an optimized gas-stripping strategy, accumulated ethanol at 1.7 g in 23 days. This yield represented a three-fold improvement in comparison to previous similar studies conducted in unicellular cyanobacteria in which, as expected, fermentation appeared to be attenuated by the O_2 produced by photosynthesis (Ehira et al., 2018). The platform described in our study produced considerably higher levels of ethanol from *Anabaena* sp. PCC 7120 cells at about 8 g in three days (two for biomass production and one for fermentation). Regardless of this progress, it becomes quite evident that achievable yields are still far away in comparison to plant feedstocks (Sanz Smachetti et al., 2018) to envision commercial competitiveness of a platform dedicated to the production of only ethanol. However, ethanol production might be considered if it occurs within a biomass biorefinery for additional products (Laurens et al., 2017), and especially, if it minimizes the use of chemicals and enzymes.

3.4. Additional cyanobacterial biomass biorefinery for recovery of protein and other cellular fractions

Both methods allowed the separation of a soluble fraction rich in sugars as a direct feedstock for ethanol. Since in the MW method protein precipitation occurred inside the cells, protein can be recovered along other water insoluble cell materials. Conversely, if extraction proceeded through the D&M method, about 50% of the total biomass crude-protein could be isolated in a water-soluble form from either strain (Fig. 6). After mild heating of these preparations, sugars remained soluble and were separated from the insoluble protein fraction (Supplementary Materials). About 20% of the dry biomass could be recovered in these fractions (Fig. 6).

There is an increasing interest to partially replace conventional sources of protein, such as soybean meal, fish meal, rice bran, etc., by microalgal and/or cyanobacterial protein for animal feeding, especially poultry and in aquaculture (Becker, 2007). Both, nutritional and toxicological determinations supported the suitability of algal biomass as a valuable feed supplement or substitute. Comparative data on biological value, digestibility coefficient, net protein utilization, and protein efficiency ratio of algal proteins suggested a quality slightly lower than those of casein or egg protein (Becker, 2007). However, poor digestibility of whole algal biomass due to the cellulosic cell wall, especially of some microalgae, is frequently one of the major drawbacks towards algal biomass used as feed (Becker, 2007). Thus, efficient (Lupatini et al., 2017; Safi et al., 2014) and especially cost-effective treatments, as those proposed in this study, are necessary to increase the feasibility of algal biomass as a partial replacement of protein- for- feed production. It has been reported that *Scenedesmus obliquus* biomass drying and cooking improved the digestibility coefficient and net protein utilization (Becker, 2007).

Cyanobacterial biomass is characterized by a low content of lipids (Hu et al., 2008). However, although not demonstrated here, the residual biomass after applying the D&M method followed by aqueous extraction of carbohydrates and protein would become enriched in lipids, which could be further biorefined for feed supplements and/or a biodiesel feedstock, as needed.

4. Conclusion

This study shows the genetic modification of carbohydrates partitioning in a filamentous cyanobacteria towards accumulation of sucrose

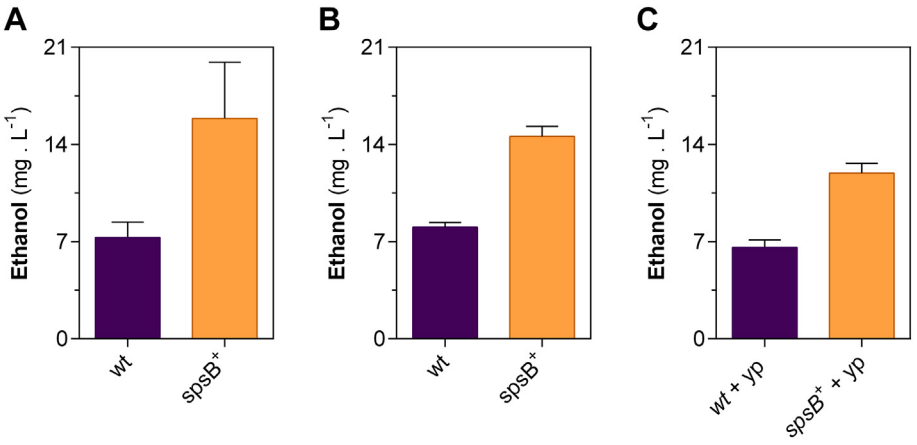

Fig. 5. Effect of *spsB* gene overexpression on biomass and sucrose production and conversion into ethanol. A–C) Ethanol production on a culture volumetric basis. Sucrose accumulation was induced at 120 mM (A) or 80 mM (B–C) NaCl. Sucrose was extracted according to the D&M (A–B) or the MW (C) methods. In (C), syrups were supplemented with yeast extract and peptone. The data represent the mean and standard deviation of four (120 mM) or two (80 mM) independent experiments.

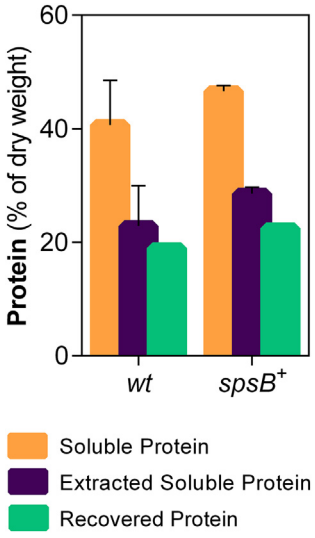

Fig. 6. Protein recovery from of *Anabaena* sp. PCC 7120 biomass. Total soluble protein in the biomass; extracted soluble protein by the D&M method; or recovered by heating. For total and extracted soluble protein, data represent the mean and range of two independent experiments and a single determination for heat-insolubilized protein.

as a readily fermentable feedstock. We optimized two methods for cost-effective preparation of concentrated sucrose syrups which could be efficiently converted into ethanol by yeasts. The D&M method followed by aqueous extraction of carbohydrates and protein, and protein recovery by short pulses of heat could keep the value of sugars, protein and lipids for different applications in the food, energy and/or other sectors of the market.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2019.01.019.

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